Nucleic Acids Research, Vol. 20, No. 21 5749–5752

Selective cleavage of pyrophosphate linkages

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Received July 9, 1992; Revised and Accepted September 28, 1992

ABSTRACT

Pyrophosphate linkages have a number of important roles in biology and are also formed chemically with great ease. They often are unwanted products, such as in the nonenzymatic oligomerization of mononucleotides. We have found that Zr^{4+}- and Th^{4+}-ions catalyze the symmetrical hydrolysis of pyrophosphate linkages. Oligonucleotide analogs linked by pyrophosphate bonds are substantially degraded in the presence of these metals, even at 0°C. Conditions are described which permit the decapping of a pyrophosphate capped oligonucleotide. Oligodeoxynucleotides can be decapped by this procedure without cleavage of phosphodiester linkages. Oligoribonucleotides are susceptible to partial hydrolysis and require purification by HPLC after decapping.

INTRODUCTION

The facility with which pyrophosphate linkages are formed whenever phosphomonoesters are activated in aqueous solution has been discussed recently by Rodriquez and Orgel (1). The capping of oligonucleotide products by a pyrophosphate linkage is an important side reaction in template-directed oligomerizations of mononucleotides, particularly under conditions where triple-helix formation is favored (2). The ease of formation of pyrophosphates is, in fact, one of the major reasons for interest in pyrophosphate-linked oligonucleotide analogs as possible precursors of the first RNA molecules (3–6). In the course of an investigation into the effects of metal-ions on such oligomerizations, we have found that the metals Zr^{4+} and Th^{4+} have substantial activity in the hydrolysis of pyrophosphate linkages.

The first investigations of metal-catalyzed transphosphorylations date from 1950 when Dimroth studied the degradation of RNA in the presence of Pb^{2+} (7). Ikenaga investigated transphosphorylations of various dinucleotides in the presence of divalent metal-ions (8). Lanthanides have been found to be more active than divalent ions (9). Complexes of metal-ions with ligands are also effective in catalytic transphosphorylation reactions (9–11). These complexes are of interest in designing artificial restriction enzymes. Metal-ion-catalyzed transphosphorylation reactions are not limited to RNA. Macrocyclic phenanthroline complexes of Cu, Co, Zn, Cd, and Pb-ions were found to catalyze the hydrolysis of DNA (12).

The metal-ion-catalyzed dephosphorylation of nucleotide triphosphates has been studied intensively (13). Metal-catalyzed hydrolysis of cAMP (14) and of the 2′,3′-cyclic phosphates of A, C, and U have been reported [9]. We are not aware, however, of reports of the catalyzed hydrolysis of internal pyrophosphate linkages. We describe here conditions under which pyrophosphate-linked polynucleotide analogs can be degraded and a pyrophosphate cap on an oligonucleotide can be removed selectively.

MATERIALS AND METHODS

Alkaline phosphatase (type III from *Escherichia coli*), nuclease S1 (from *Aspergillus oryzae*), and $(pdT)_5$ were purchased from Sigma Chemical Company. Phosphodiesterase I (from *Crotalus adamanteus* venom) was purchased from P-L Biochemicals. The oligoribonucleotide $A(pA)_8$ was purchased from Collaborative Research. Poly(C), poly(A), poly(U) and poly(G) were from Sigma. The preparations of the acyclic nucleotide analogs pG̃p and pC̃p have been described (4, 15). The cyclic pyrophosphates of pC̃p and pG̃p were prepared following a procedure described for the synthesis of cpC̃p (6). Oligomers of pC̃p and pG̃p were prepared using the thermal oligomerization method described by Tohidi and Orgel (16). The cyclic monomers (1 μmol), dissolved in 10 μl 0.1 M imidazole (pH 6.5 with HCl), were heated in open tubes (12×75 mm) for three days over P_2O_5 at 85°C. The oligomeric mixtures were used for hydrolytic studies without further purification.

Oligomers (0.0126 M, monomer-equivalent) and 0.025 M Zr^{4+} or Th^{4+} in 0.4 M Bis tris (pH 6.5) were incubated at 0°C. After 7 days, the reactions were stopped by adding 1 μl 1 M EDTA (pH 9). Similar incubations were carried out at 0°C with poly(C), poly(A), poly(U) and poly(G). Analysis was performed by HPLC on RPC-5 (0.02 M NaOH with a linear gradient of 0–0.04 M $NaClO_4$). Adenosine 5′-phosphoimidazolide (ImpA) was prepared as described previously (17). The synthesis of A(5′)ppdT$(pdT)_4$ (I) was carried out using a modification of the procedure of Chu and Orgel (18). One ODU of $(pdT)_5$ was dissolved in 10 μl of 0.2M HEPES (pH 7.0) containing 0.2 M $MnCl_2$ and 0.1 M ImpA. After 4 h at 50°C the reaction was quenched by addition of 4 μl EDTA (1 M, pH 9.0). Analysis

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by HPLC on RPC-5 indicated a 77% conversion of the pentamer to the pyrophosphate-capped product **I**. The product was isolated from the RPC-5 column and the pH of the fraction was adjusted to 8.0 with 1 M HCl. The identity of the product was confirmed by enzymatic degradation. The pentamer $(pdT)_5$ and the pyrophosphate **I** were each (0,02 ODU) incubated with alkaline phosphatase (0.1 unit) at pH 8.0 for 4 h at 37°C. Analysis by HPLC showed that only $(pdT)_5$ was dephosphorylated. Incubation of **I** with nuclease S1 (100 units) in 0.036 M sodium acetate buffer (pH 4.5) containing 0.07 mM $ZnSO_4$ and 0.05M NaCl for 2 h at 37°C resulted in degradation to A(5')ppdT. Incubation with phosphodiesterase I (0.4 units in 0.04 M $MgCl_2$ and 0.1 M Tris−HCl (pH 9.0) for 4 h at 37°C resulted in complete hydrolysis of both pentamer and its pyrophosphate.

Reaction mixtures were prepared by adding 63 μl of the isolated pyrophosphate-capped oligomer (0.01 ODU) to 10 μl of Bis−Tris (3 M, pH 6.5 or 6.0) or NaOAc (3 M, pH 4.5, 5.0 or 5.5), together with 3 μl of 1 M $ZrCl_4$ or $Th(NO_3)_4$ or water. The final concentration of the metal-ion was 0.04 M. Incubation was carried out at 0°C or 50°C. Similar incubations were carried out at 50°C with $A(pA)_8$ in the presence of Zr^{4+}. After addition of 6 μl of EDTA (1 M, pH 9.0), analysis was performed by HPLC on RPC-5.

RESULTS AND DISCUSSION

Incubation of pyrophosphate-linked, atactic oligomers of pG̃p (Figure 1) and pC̃p in the presence of Zr^{4+} or Th^{4+} at pH 6.5 and 0°C resulted in a symmetrical hydrolysis of the pyrophosphate linkage, yielding a series of shorter oligomers as well as monomer (Figures 2 and 3). Oligomers with chain-lengths longer than 10 decreased, in the presence of Th^{4+}, from about 30% to 1% after one week in the case of oligo(pC̃p) and to 6−8% in the case of oligo(pG̃p). Zr-ions were slightly less effective in the hydrolysis than Th-ions. Control reactions without metal showed no degradation.

Although many examples of metal-catalyzed hydrolysis of RNA are known, these have generally been conducted at elevated temperatures. When we treated polyribonucleotides with Zr^{4+} or Th^{4+} at 0°C, we found no detectable degradation after 7 days. Since there was a large difference between the degree of hydrolysis of pyrophosphate-linked analogs and polyribonucleotides in the presence of Zr^{4+} or Th^{4+} at 0°C, we tested the use of Zr or Th in selectively removing pyrophosphate caps of oligonucleotides.

To determine the minimal conditions necessary to hydrolyze a pyrophosphate cap, we synthesized and studied the degradation of A(5')ppdT$(pdT)_4$. Incubation of the capped oligomer in the presence of Zr^{4+} resulted in 59% hydrolysis to the free pentamer after 7 days at pH 6.5 and 0°C (Table 1). The limited

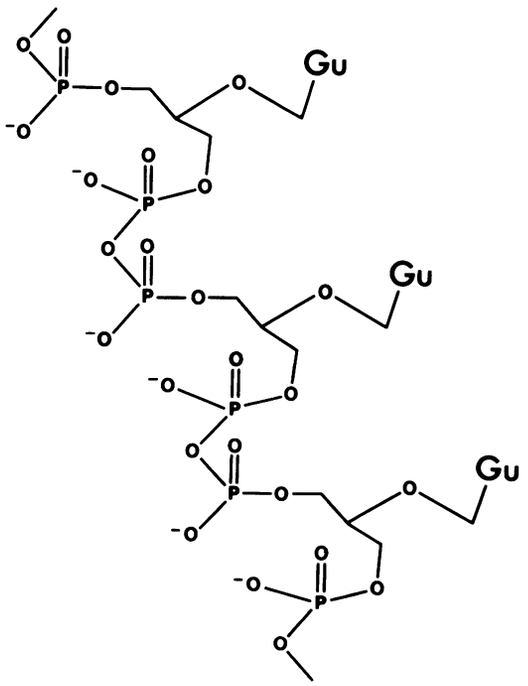

Figure 1. The pyrophosphate-linked backbone of an acyclic polynucleotide analog, oligo(pG̃p). The chain is presumed to be atactic.

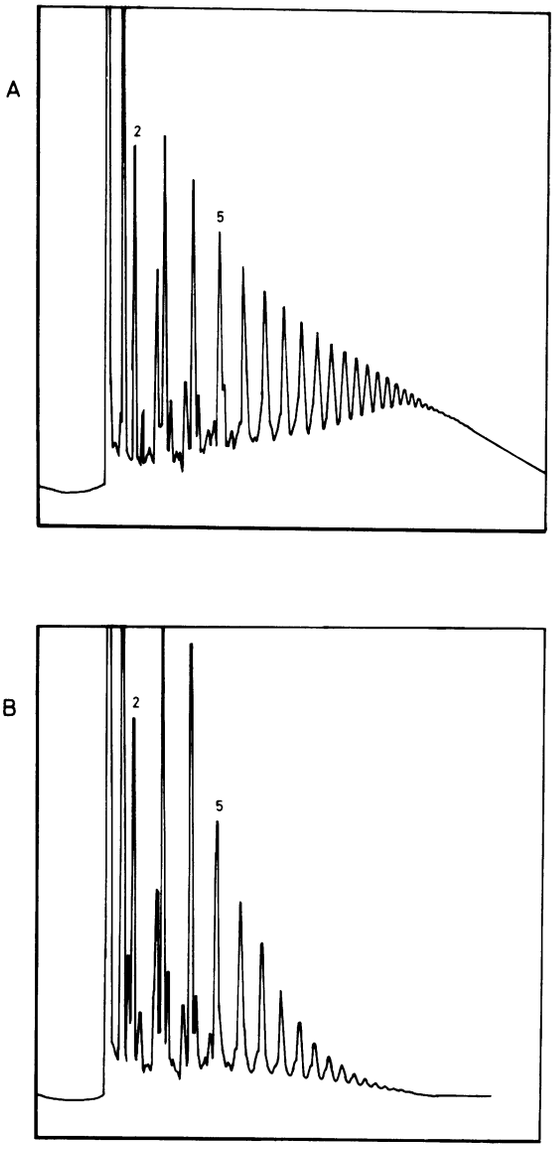

Figure 2. HPLC analysis showing partial degradation of pyrophosphate-linked oligomers of pG̃p. **A**, After incubation at 0°C and pH 6.5 for 7 days. **B**, After incubation in the presence of Th^{4+} at 0°C and pH 6.5 for 7 days.

degradation in the presence of Th^{4+} was caused by the precipitation of the Th^{4+}-complex with A(5')ppdT(pdT)$_4$. To determine the conditions essential for a complete hydrolysis of the pyrophosphate cap, reactions were performed in the presence of Zr^{4+} at 50°C and at various pH values. An example of such a decapping reaction is illustrated in Figure 4. In Figure 5 the initial rate – pH profile for the decapping of A(5')ppdT(pdT)$_4$ at 50°C is shown. The max. rate of hydrolysis of the pyrophosphate cap lies between pH 4.5 and 5.5. These conditions, while suitable for the decapping of an oligodeoxynucleotide, produce substantial degradation of high molecular weight polyribonucleotides. To determine the sensitivity of short oligoribonucleotides to the procedure, we examined the rate of hydrolysis of a single, internal phosphodiester linkage of A(pA)$_8$. In the initial rate – pH profile for the hydrolysis of A(pA)$_8$ in the presence of Zr^{4+} at 50°C (Figure 6), a sharp decrease in rate of hydrolysis is seen in the acid region. Comparison of the data of Figures 5 and 6 suggests an optimal pH of 4.5 at 50°C. The data of Table 2 illustrate that a one-hour treatment at pH 4.5 and 50°C would

Table 1.

Hours	Decapping of A(5')ppdT(pdT)$_4$ in the presence of M^{4+} (% conversion to (pdT)$_5$	
	Zr^{4+}	Th^{4+}
24	19	5
48	30	8
73	37	13
168	59	33

Conditions: 0.01 ODU oligomer, 0.04 M $ZrCl_4$ or $Th(NO_3)_4$ in 0.4 M Bis–tris (pH 6.5) at 0°C.

Table 2.

Hours	Zr^{4+} catalyzed hydrolysis (%)	
	Degradation of A(pA)$_8$	Decapping of A(5')ppdT(pdT)$_4$
1	6	84
2	10	91
4	19	96

Conditions: 0.01 ODU A(pA)$_8$ or A(5')ppdT(pdT)$_4$, 0.04 M $ZrCl_4$ in 0.4M NaOAc (pH 4.5) at 50°C.

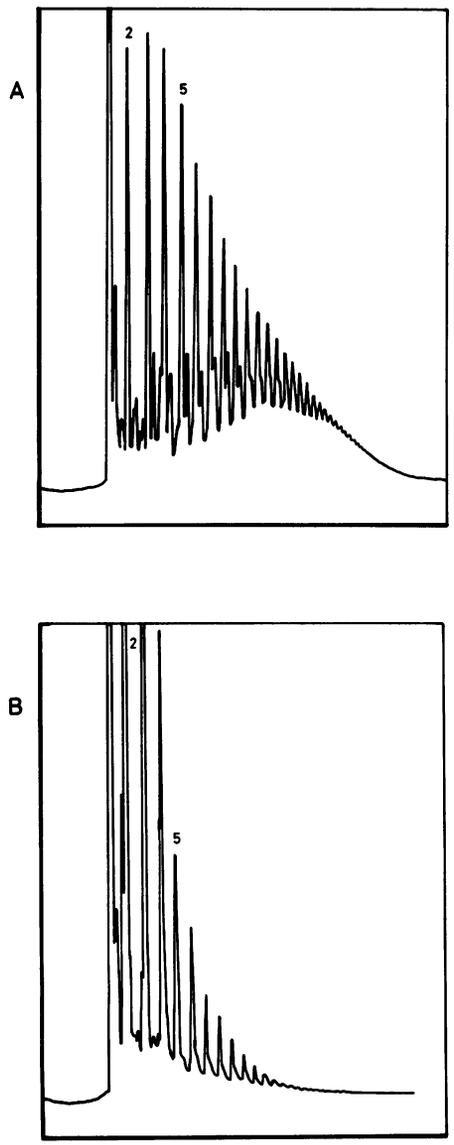

Figure 3. HPLC analysis showing partial degradation of pyrophosphate-linked oligomers of pCp. **A**, After incubation at 0°C and pH 6.5 for 7 days. **B**, After incubation in the presence of Th^{4+} at 0°C and pH 6.5 for 7 days.

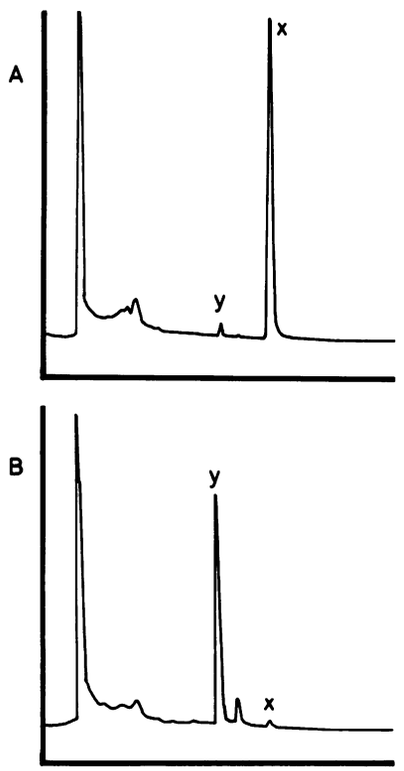

Figure 4. Decapping of A(5')ppdT(pdT)$_4$ in the presence of Zr^{4+} at pH 6.5 and 50°C. **A**, HPLC analysis at zero time. **B**, HPLC analysis after 16 h incubation. X = A(5')ppdT(pdT)$_4$, Y = (pdT)$_5$. Between X and Y a small peak is visible in **B** corresponding to about 5% dephosphorylation of (pdT)$_5$ to T(pdT)$_4$.

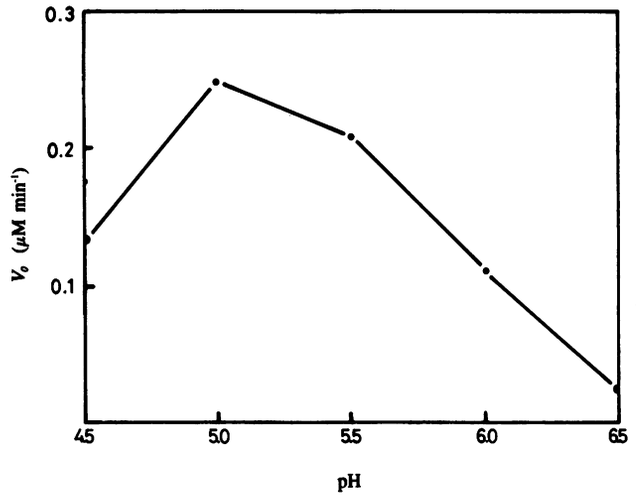

Figure 5. Dependence of the initial rate v_0 on the pH for the Zr^{4+} promoted decapping of A(5')ppdT(pdT)$_4$ at 50°C.

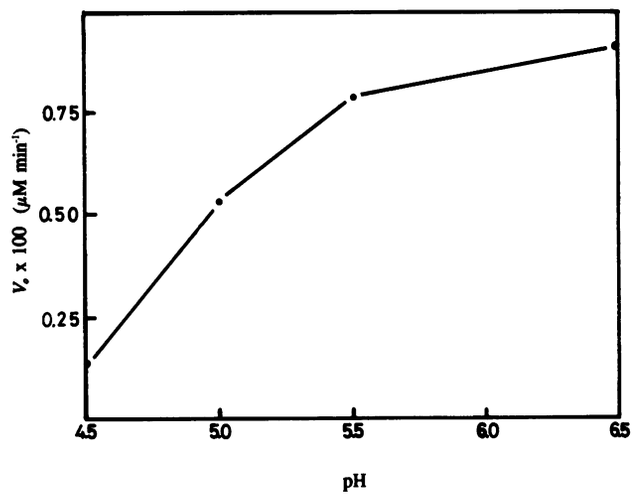

Figure 6. Dependence of the initial rate v_0 on the pH for the Zr^{4+} promoted hydrolysis of A(pA)$_8$ at 50°C.

be adequate to obtain more than 80% decapping and would produce less than 10% degradation of a sensitive oligoribonucleotide. The decapped oligoribonucleotide can consequently be isolated easily by means of preparative HPLC.

The complexities of metal ion-catalyzed hydrolysis have been well documented for nucleoside triphosphates (19), but do not permit a direct comparison with the present results, which may depend upon a different mechanism. Nevertheless, the optimal conditions for decapping which we have sought to define may prove to be of some practical value to other workers.

ACKNOWLEDGEMENTS

This work was supported by US National Aeronautics and Space Administration grant NGR 05067. We thank A.H.Hill for a gift of RPC-5.

ABBREVIATIONS

(pdT)$_5$, pentathymidylic acid;
A(pA)$_8$, adenylyl(3',5') octa-adenylic acid;
pG̃p, 9-[(1,3-diphospho-2-propoxy)methyl]guanine;
pC̃p, 1-[(1,3-diphospho-2-propoxy)-methyl]cytosine;
cpC̃p, the cyclic pyrophosphate of pC̃p.

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